

MEDICAL KITField of the Invention

The present invention relates to a medical kit for qualitative or quantitative determination of a drug in a biological fluid as well as a method therefor.

5 Background of the Invention

10 The determination of an effective therapeutic dose of a drug is often difficult, and this is mainly due to a great individual variation with respect to drug absorption and metabolism. An individual variation is also commonly seen due to the effect of e.g. food, body weight and possible interaction with other drugs present. Although this individual variation is not of major concern for some drugs, e.g. aspirin, it is nevertheless of importance for most other drugs. Indeed, with very potent
15 drugs, particularly those used in treatment of cancer and heart diseases, a non-optimal dose may yield highly detrimental, even fatal, results. Consequently, a plethora of different methods have been developed in order to provide a qualitative or quantitative determination of drugs
20 in biological fluids.

Many of the present methods used for the determination of drugs in biological fluids are based on rather complex procedures, such as those requiring HPLC, GC or GC/MS equipment. Evidently, such procedures are often inconvenient for both individual and rapid drug monitoring
25 directly at the point-of-care, such as at home or basically at any location in- or outside a healthcare facility. As is well known, various medical kits which allow drug monitoring at the point-of care have therefore been

developed. Among the public, the most well known of these kits is probably the pregnancy test Clearblue®.

Said medical kits are usually based on some kind of immunoassay. Furthermore, they normally require lateral
5 flow or diffusion through a carrier or membrane in order to function properly (see e.g. EP 833 159). However, the main problem associated with medical kits requiring significant lateral flow, or diffusion, is that non-specific binding and interaction between the present reagents, or
10 analytes, and the carrier is often observed. Such non-specific binding and interaction reduces the reliability of the analysis.

Moreover, there are ongoing demands in the art to provide a medical kit which allows the following:
15 rapid determination of drug levels in a biological fluid, where rapid typically means less than 60 minutes from biological fluid sampling to result;
simple and reliable use thereof, also for individuals not having any particular medical knowledge;
20 low energy input and/or no required controlled heating when used;
low or negligible temperature dependence;
storage stability; and
manufacturing at low costs, thus making the medical kit
25 attractive for use also in third-world countries.

Disclosure of the Invention

According to the present invention, there is now provided a novel medical kit which overcomes the problem with non-specific binding and interaction, thus providing
30 a highly reliable method for qualitative or quantitative determination of a drug in a biological fluid. Also the aforementioned demands in the art are well met by the present invention. More specifically, the present inven-

tion relates to a medical kit for qualitative or quantitative determination of a drug in a biological fluid, said medical kit comprising:

- i) a first part coated with a drug conjugate; and
- 5 ii) a second part which contains a labelled anti-drug antibody and is adapted for receiving said biological fluid, wherein said labelled anti-drug antibody is labelled with gold material and/or latex particles. Preferably, said drug conjugate is
- 10 a conjugate between said drug and a protein, peptide, polyamine, ceramide, alkyl chain or carbohydrate. Most preferably, said protein is selected from rabbit serum albumin (RSA), bovine serum albumin, ovalbumin, gamma globulin and thyro globulin.

- 15 In an embodiment of the invention the latex particles are coloured. Coloured latex particles are commercially available from a number of sources such as Polymer Laboratories Ltd., Essex Road, Shropshire, England and Bangs Laboratories, US. Examples of latex particles are
- 20 "Dyed polymer microspheres" from Bangs Laboratories Inc., US, catalogue No. DS03B, 0,5-0,9 μm diameter. These microsphere particles have an intense blue colour. They bind proteins by adsorption. Polymer Laboratories Ltd., Shropshire, England also provides latex particles for antibody
- 25 labelling, for example PL-Latex Plain HiDye™ Blue 400nm, Catalogue No. 6004-412.

- In another embodiment of the invention the gold material used as labelling material is colloidal gold. The colloidal gold may comprise metallic gold as well as gold
- 30 ions. The novel use of gold-labelled and/or coloured latex particles labelled antibodies for detection of analytes in a solid phase/liquid setting according to the invention have many advantages over the prior art.

Firstly, the use of colloidal gold instead of enzyme as label makes this embodiment substantially unaffected by normal temperature fluctuations, which is especially advantageous from a reproducibility, storage as well as user aspect. Secondly, this embodiment provides an extremely rapid analysis (5-15 minutes from sampling to result), since the colour development requires no additional step involving a substrate for a labelling enzyme. Indeed, the use of a gold material such as colloidal gold is particularly preferred according to the present invention.

Preferably, said drug is selected from antihypertensive, antiviral, antimicrobial, antifungal, antiinflammatory, antitumor and psychopharmaceutical agents, including corticosteroids, mono- to oligosaccharides, vitamins, provitamins and hormones.

It is particularly preferred that said drug has a molar weight in the range of from 50 to 6000 g/mol, preferably from 100 to 1000 g/mol.

Typically, said drug is selected from lisinopril, amlodipine, captopril, enalapril, enalaprilat, ketotifen, sildenafil and fluoxetine.

In the present medical kit, said second part is preferably a container which either contains a solution of said labelled anti-drug antibody or has its interior at least partially coated therewith. Typically, said container is tube-shaped.

As an example, said first part typically has the shape of a stick, strip, paddle or plate. The shape of a Nunc-Immuno™ Stick (*vide infra*) is particularly preferred.

As for the preferred material of said first part, its surface is typically of a material selected from

polystyrene, polypropylene, nitrocellulose material chemically treated for maximum binding.

The material below the surface may be of either the same or a different material as that of the surface. The surface of said first part may thus be either completely integrated with the underlying material, i.e. said first part is made of a single type of material, or is a layer applied on virtually any kind of useful material. Preferably, the surface of the first part is that used for the surface of a Nunc-Immuno™ Stick. In a preferred embodiment, said first part is a Nunc-Immuno™ Stick, as exemplified hereinbelow. By using the Nunc-Immuno™ Stick as the first part (i) mentioned above for the solid phase immunoassay according to the invention surprisingly good results are obtained. It is specially preferred due to the advantage of a rapid near-patient medical kit for monitoring drug levels in biological fluids of patients.

Moreover, the present invention also relates to a method for qualitative or quantitative determination of a drug in a biological fluid, wherein a first part coated with a drug conjugate, i.e. a first part i) as defined above, and a labelled anti-drug antibody, wherein said labelled anti-drug antibody is labelled with gold material and/or latex particles, are utilised.

In accordance with said method, said first part is preferably placed inside a second part which contains a labelled anti-drug antibody and is adapted for receiving said biological fluid, i.e. a second part ii) as defined above, wherein said second part contains said biological fluid, and wherein said first part is removed from said second part after a predetermined period of time.

Said predetermined period of time is typically in the range of from 5 to 60 minutes.

According to said method, said first part is subsequently, i.e. after the aforementioned steps have been performed, developed in order to bring about a potential colour change indicating the level of said drug in said biological fluid.

When using colloidal gold when labelling the anti-drug antibody in connection with the present invention extremely good results are obtained, ie dark brown-red colour, see figure 4. Gold can also be regarded as a comparable cheap substance to use for labelling, especially in comparison with certain expensive purified enzymes often used as labelling material.

The kit according to the invention has the advantage that it can be used by an unskilled person for self-monitoring of a drug in a biological sample, ie urine, blood or saliva, easily without the aid of any additional instrumentation.

Usually, said method in its entirety is performed at ambient temperature. Typically, said biological fluid is blood, serum, urine or saliva.

As used herein, the expression "drug" comprises any substance which may yield or relate to a physiological response or condition, particularly when administered to a living organism. Thus, for the purposes of the present invention, also metabolites (active or non-active), pro-drugs and antigens are considered as drugs.

The present invention is further illustrated by the following non-limiting examples together with the accompanying figures.

Description of the figures

Fig. 1 depicts a standard curve for an HRP labelled anti-lisinopril antibody present at a concentration of 25

ng/ml. The detected levels of lisinopril (pg/ml) were monitored at a wavelength of 450 nm.

Fig. 2 depicts titration of HRP labelled anti-lisinopril antibody against sticks coated with either an RSA-lisinopril conjugate or RSA only.

Fig. 3 depicts the ability of the present medical kit to both detect and distinguish between different levels of lisinopril in human whole blood by utilising said first part coated with an RSA-lisinopril conjugate. An HRP labelled anti-lisinopril antibody was used for detection.

Fig. 4 depicts titration of gold-labelled (Au^{3+}) rabbit anti-lisinopril antibody against sticks coated with either an RSA-lisinopril conjugate or RSA only.

Fig. 5 depicts detection of different levels of lisinopril in human whole blood by utilising said first part coated with an RSA-lisinopril conjugate and said second part containing gold-labelled rabbit anti-lisinopril antibody for detection.

Examples

Materials and procedures:

Drug conjugates were prepared essentially in accordance with the methodology presented in "Bioconjugate Techniques", Ed. Hermanson, G., Academic Press, USA, 1996. The preparation of the anti-drug antibodies was essentially performed by following the procedures set forth in "Synthetic polypeptides as antigens", Eds. van Regenmortel, M.H.V., Briand, J.P., Muller, S. and Plaue, S., Elsevier, Holland, 1988. For preparation of anti-drug antibodies in general, see also "Practice and theory of enzyme immunoassays", Tijssen, P., Elsevier Publishing, Holland, 1985, chapters 5 and 12. The entire teachings of all the publications referred to above are incorporated

herein by reference.

Principle of the present invention when using the Nunc immunostick as the first part (i) mentioned above:

In this assay the analytes present in the biological
5 fluid competes with the standard analyte which is immobi-
lised on the sticks for binding with the enzyme-labelled
, preferably gold-labelled, anti-analyte antibody. The
procedure will be described with reference to lisinopril.
The Nunc paddles are coated with the protein-lisinopril
10 conjugate. This binds strongly by non-covalent interac-
tion. Excess protein-drug conjugate is washed away and
any free binding sites on the sticks are blocked by incu-
bation with a protein- and detergent-containing buffer
e.g., casein and Tween-20. After blocking, the sticks are
15 washed in water, dried and can be stored under dry condi-
tion until further use. In the test, the sticks are
placed in a tube containing a dilution of the enzyme-
labelled, preferably gold-labelled, anti-lisinopril anti-
body and a few drops of the biological fluid being
20 tested. After 5-10 minutes incubation to allow for the
antigen-antibody reaction, the sticks are washed in tap
water to remove excess labelled antibody. If the bio-
logical fluid does not have the drug, then most of the
labelled antibody will bind to the drug immobilised on
25 the sticks. This binding will reveal itself directly, in
the case of gold-labelled antibody, as the gold-antibody
has intense reddish colour. In the case of enzyme-
labelled antibody, the binding of the antibody is re-
vealed by the addition of a substrate (TMB in the case of
30 horseradish peroxidase). The enzyme-labelled antibody,
which is now bound to the drug immobilised on the stick,
will convert the soluble substrate into an insoluble blue
coloured product that will be deposited on the surface of

the stick. The sticks are rinsed in water to remove excess substrate solution and dried. If the biological fluid contains the drug, then this will compete with the immobilised drug for binding with the antibody conjugate.

5 This means that less antibody conjugate will bind to the drug immobilised on the sticks. Hence, the sticks will be coloured less intensely. The more free drug present in the biological fluid the less the colour intensity would be. In practice, an antibody conjugate dilution is selected that will give intense colour at or below the cut
10 off concentration and no colour or faint colour at the "positive" concentration, e.g., for lisinopril the cut off chosen is 50 ng/ml. At this concentration or below sufficient antibody conjugate will bind to give intense
15 colour, while at 100 ng/ml the colour intensity is faint or absent.

Alternatively, an indirect standard immunoassay procedure may be used instead of directly labelling the anti-drug antibody (e.g., rabbit anti-lisinopril anti-
20 body). In this embodiment one can use a labelled second antibody, e.g., enzyme-labelled, or gold-labelled goat anti-rabbit IgG antibody. In this case, the assay will be performed as before but with the addition of an extra step. Hence, the coated strips will be incubated with the
25 anti-drug antiserum (rabbit anti-lisinopril) for 5-10 minutes, followed by washing in water for 10 seconds, incubation with labelled goat anti-rabbit IgG for another 5-10 minutes, washing and reading for gold-labelled antibody directly or after the addition of the substrate in
30 the case of enzyme labelled antibody.

Yet another variation to the indirect test is to label the anti-drug antibody with biotin and using gold-

labelled or enzyme labelled avidin or streptavidin in place of the second antibody.

Preparation of Rabbit Serum Albumin-Lisinopril conjugates by 2-step glutaraldehyde method:

- 5 1. 10 mg of rabbit serum albumin is dissolved gently in 1 ml of PBS (phosphate buffered saline: 0.02M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4 containing 0.15M NaCl).
2. 1 ml of freshly prepared 1 % (w/v) glutaraldehyde is added to the protein dropwise while stirring. Leave
- 10 stirring for 2 hours at room temperature.
3. Excess glutaraldehyde is removed by gel filtration in PBS, using either Sephadex G-25F or Sephadex G-50M. A column with at least 20 ml of gel (recommend 1.5 x 20cm) will be needed. Monitor protein peak at 280 nm.
- 15 4. Fractions containing the protein are pooled and lisinopril solution in PBS is added. A molar ratio of lisinopril: RSA of 200: 1 is used. Incubation while mixing for 24 hours at room temperature.
5. Dialyse vs. PBS for 48 hours at 4°C.
- 20 6. Optical density is measured at 280nm. Conjugate concentration using the extinction coefficients for a 1 mg/ml protein concentration: 0.67 at 280 nm is worked out.

Sodium azide is added to 0.1 % (w/v) and aliquots are

25 stored at -20°C.

Preparation of IgG-Horseradish peroxidase conjugates (HRP-IgG) by 2-step glutaraldehyde method:

1. 10 mg of HRP is dissolved gently in 1 ml of PBS (phosphate buffered saline: $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4
- 30 containing 0.15M NaCl).
2. 1 ml of freshly prepared 1 % (w/v) glutaraldehyde is added to the enzyme dropwise while stirring. Leave stirring for 2 hours at room temperature.

3. Excess glutaraldehyde is removed by gel filtration in PBS, using either Sephadex G-25F or Sephadex G-50M. A column with at least 20 ml of gel (recommend 1.5×10 cm) will be needed. Monitor enzyme peak at 403 nm.
- 5 4. Fractions containing the enzyme are pooled and antibody solution in PBS is added. A molar ratio of HRP: IgG of 4-6: 1 is used. The proteins are incubated while mixing for 24 hours at room temperature or 4°C if IgG is labile.
- 10 5. Dialyse vs. PBS for 48 hours at 4°C .
6. O.D. is measured at 403 nm and 278 nm. HRP and IgG concentrations in the conjugate using the extinction coefficients for a 1 mg/ml protein concentration are worked out:
- 15 $\text{HRP}_{403\text{nm}} = 2.2$, $\text{HRP}_{278\text{nm}} = 0.75$, $\text{IgG}_{278\text{nm}} = 1.4$.
7. BSA is added to 10 mg/ml, Tween-20 to 0.05 % (w/v) and a preservative: 0.005 % thiomersal or still better 0.02 % (w/v) bromo-nitro-dioxane (BND). Aliquot are stored at -20°C .
- 20 Note:
HRP-drug conjugates are made following the same procedure, using drug: HRP molar ratio of 5:1.
Rabbit anti-lisinopril antibody was gold-labelled by British Biocell International, Cardiff, Wales, Britain.
- 25 Sensitivity evaluation:
The interior walls of conventional microtitre wells were coated with an RSA-lisinopril conjugate by adding a solution of said conjugate ($1 \mu\text{g/ml}$; 0.1 ml) to said wells followed by incubation for 1 h at 37°C . After washing with wash buffer (10 mM sodium phosphate buffered saline, pH 7.4 containing 0.05 % sodium azide and 0.05 % Tween-20, PBS-T), 250 μl /well, 3 times, the wells were
- 30

incubated with varying amounts of lisinopril in human whole blood (25 μ l) in the presence of HRP labelled anti-lisinopril antibody (25 ng/ml in wash buffer, 100 μ l/well) for 30 min at room temperature. After washing with wash buffer the wells were incubated with the substrate, tetramethylbenzidine solution, 100 μ l/well for 30 min at room temperature. Colour development was quenched by addition of 2 M sulphuric acid, 100 μ l/well and the colour intensity was measured at a wavelength of 450 nm by use of a Dynex microtitre plate reader. The results are depicted in Fig. 1. Since a lisinopril level as low as 100 pg/ml could be detected, this experiment verified that the sensitivity of the assay system of the present invention is very high.

15 Evaluation of possible non-specific binding:

Sticks coated with RSA-lisinopril conjugate were prepared by submerging a Nunc-Immuno™ Stick (manufactured by Life Technologies, UK) into a solution of RSA-lisinopril conjugate (5 μ g/ml) in carbonate buffer (pH 9.5), followed by incubation for 1 h at 37°C. Phosphate buffered saline (PBS; pH 7.4) may optionally be used as solvent, and the incubation may alternatively be performed for 24 h at room temperature. After the incubation, the stick was washed in tap water and blocked in PBS containing 0.2% casein, 0.05% Tween-20 and 0.05% NaN₃ (preservative) for 1 h at room temperature. Then it was rinsed once in tap water, dried at room temperature and stored in a dry environment until further use thereof.

The above describes a generally applicable coating procedure, and sticks coated with RSA were prepared in complete accordance therewith.

Moreover, some details of the coating procedure deserve additional comments. Firstly, a direct coupling of the drug to a stick would not yield a useful product, since the fairly low molecular weight of the drug would make it nearly impossible for an antibody to directly bind to the immobilised drug. For this reason, sticks were instead coated with a drug conjugate, preferably a protein-drug conjugate such as RSA-lisinopril. Conjugates between a drug and a polyamine or carbohydrate are equally useful. Secondly, it was also found that i) coating with a high amount of conjugate, and ii) a high drug/protein ratio in the conjugate were absolutely crucial for attaining high sensitivity. Typically, the concentration of the conjugate in the above coating solution should be in the range of 1-5 $\mu\text{g/ml}$, whereas a useful drug/protein ratio in the conjugate should be from 10:1 to 200:1.

The sticks prepared as above were then inserted into tubes containing 10% (v/v) human whole blood in phosphate buffered saline (0.5 ml), containing 0.05% Tween-20, 0.2% casein and 10 mM EDTA (pH 7.4), as well as various concentrations of HRP labelled anti-lisinopril antibody. After incubation for 5 min at room temperature, each stick was removed, rinsed in tap water for 10 seconds and then inserted into tubes containing tetramethylbenzidine substrate for 5 minutes, after which it was rinsed as before, dried and photographed. The results are depicted in Fig. 2, and they confirm that non-specific antibody binding to the sticks is insignificant in comparison with the very high degree of specific antibody binding. A major prerequisite for the versatility of the present invention is thereby provided. For practical purposes, the most suitable colour intensity was obtained when the concen-

tration of the HRP labelled anti-lisinopril antibody was 250 ng/ml.

Detection of lisinopril in human whole blood:

- Sticks coated with RSA-lisinopril conjugate as above
- 5 were submerged into a mixture (500 μ l) of HRP labelled anti-lisinopril antibody (250 ng/ml; 400 μ l; in phosphate buffered saline (0.5 ml), containing 0.05% Tween-20, 0.2% casein and 10 mM EDTA (pH 7.4)), and human whole blood (100 μ l) containing various concentrations of lisinopril.
- 10 After incubation for 5 min, the sticks were removed, rinsed in running tap water and developed with tetramethylbenzidine as above. The results are shown in Fig. 3, and they clearly illustrate a marked colour intensity difference between the various evaluated lisinopril concentrations. Note in particular the clear difference in
- 15 colour intensity between the blood samples containing 50 ng/ml and 100 ng/ml, respectively, of lisinopril. Since these concentrations cover the effective therapeutic range of this drug, the results *supra* should be of considerable medical relevance.
- 20

Evaluating the binding specificity of gold-labelled rabbit anti-lisinopril antibody:

- Colloidal gold is made up of clusters of gold ions. Colloidal gold is not a molecule of defined structure,
- 25 but suspended particles with some of the properties of metallic gold. In solution, colloidal gold adopts a negative charge, thought to arise from gold iodine chloride (AuICl_2) which make up the surface of the particle. Because of this negative charge, colloidal gold has an affinity for many proteins, which tend to have a positive
- 30 or neutral charge at physiological pH. This allows adsorption of antibodies to colloidal gold particles. The colloidal gold particles themselves have intense red col-

our. Hence, when the gold-labelled antibody is concentrated at the site of the corresponding antigen, you get intense brown-reddish colour whose intensity will obviously depend on the number of antibody molecules bound.

5 Colloidal gold labelled antibodies are commercially available to certain antigens. For example, colloidal gold labelled anti-drugs of abuse antibodies can be bought from many companies such as British Biocell International (BBI), Cardiff, Wales, United Kingdom. In the
10 present invention the labelling is performed by BBI using their propriety techniques. This is important as each antibody may require certain means for labelling. It is however possible to perform the gold labelling directly by using a suspension of colloidal gold that can be purchased from BBI.
15

Sticks coated with either RSA-lisinopril conjugate or RSA only were prepared as described above. They were then incubated for 20 min at room temperature with gold-labelled rabbit anti-lisinopril antibody (200 µl; 0.2 or
20 0.1 optical density units/ml; in phosphate buffered saline (0.5 ml), containing 0.05% Tween-20, 0.2% casein and 10 mM EDTA (pH 7.4),) in the presence of human whole blood (50 µl). After the incubation the stick was removed and rinsed with tap water for 10 seconds, whereby potential colour (red) development was immediate. The results
25 are shown in Fig. 4, and they evidence a very high binding specificity also for this system. The solution of gold-labelled rabbit anti-lisinopril antibody having an optical density of 0.2/ml was most preferred for further
30 use.

Detection of lisinopril in human whole blood by utilising gold-labelled antibody:

Sticks coated with RSA-lisinopril conjugate as above were submerged into mixtures of gold-labelled rabbit anti-lisinopril antibody (200 μ l; 0.2 optical density units/ml; in phosphate buffered saline (0.5 ml), containing 0.05% Tween-20, 0.2% casein and 10 mM EDTA (pH 7.4), and human whole blood (50 μ l), the latter containing lisinopril at the concentrations 0, 20 and 100 ng/ml, respectively. After incubation for 15 min, the sticks were removed and rinsed in running tap water for about 10 seconds, providing immediate colour development. The results are shown in Fig. 5, and they clearly illustrate a distinguishable colour intensity difference between the evaluated lisinopril concentrations.

The characteristics of the present embodiment which utilises gold-labelled anti-drug antibody require some additional comments. Firstly, the use of gold instead of enzyme as label makes this embodiment substantially unaffected by normal temperature fluctuations, which is especially advantageous from a reproducibility, storage as well as user aspect. Secondly, this embodiment provides an extremely rapid analysis (5-15 minutes from sampling to result), since the colour development requires no additional step involving a substrate for a labelling enzyme. Indeed, this embodiment is particularly preferred.

According to preliminary experiments, the optional use of anti-drug antibody labelled with latex particles (e.g. carboxy activated beads, Bangs Laboratories Inc., U.S.A.) yields a product having properties nearly identical to those of the gold-labelled anti-drug antibody system disclosed above.

To summarise, the present medical kit is typically used in practise as follows:

- A solution of about 200-500 μ l of the labelled anti-drug antibody is added to a second part, normally a tube, followed by addition of about 50-100 μ l of the tested biological fluid, normally blood. After incubation for 1-3 minutes, a first part, i.e. a coated stick prepared as above, is placed inside the tube and incubated for 5-10 minutes. The stick is then removed, rinsed in tap water for 10 seconds and developed. The concentration of the solution of the labelled anti-drug antibody should preferably be selected so that test samples containing a drug level below the effective therapeutic dose yield a fairly intensely stained stick, whereas test samples containing a drug level within the effective therapeutic dose should yield only a weakly stained, or even unstained, stick.
- The entire procedure is normally performed at ambient temperature and requires less than 60 minutes from blood sampling to result. Hence, it should be evident that the concept of the present invention provides a significant contribution to the art.